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Energetics of cyclodextrin-induced dissociation of insulin

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Abstract The energetics of dissociation of bovine insulin in aqueous solution have been investigated by sensitive dilution microcalorimetry. Cyclodextrins increase dissociation of insulin oligomers in a manner consistent with their interaction with protein side chains. For example, assuming monomer-dimer equilibrium, in the absence of cyclodextrins the calorimetric dilution data (25 °C, pH 2.5) indicate a dimer dissociation constant (K_{diss}) of about 12 μM and an endothermic dissociation enthalpy (ΔH_{diss}) of +41 kJ mol^{-1}. Addition of methyl-β-cyclodextrin (up to 200 mM) makes dissociation significantly more endothermic ($\Delta H_{diss} = 79$ kJ mol^{-1}) and reduces the apparent dimer dissociation constant by more than two orders of magnitude ($K_{diss} \approx 1.7$ mM). Qualitatively similar results are observed with α-cyclodextrin and other β-cyclodextrin derivatives. Cyclodextrin-induced insulin dissociation is also observed at pH 7.4.

Key words Cyclodextrin · Insulin · Dimer · Dissociation · Microcalorimetry · Thermodynamics

Introduction

Insulins are known to occur in a variety of aggregation (oligomer) states in solution depending on concentration, pH, temperature, Zn^{2+} concentration and other ionic conditions (Blundell et al. 1972; Bi et al. 1984), and their aggregation states can potentially affect their use in therapeutic situations. Based on previous observations on interaction of cyclodextrins with globular proteins (Cooper 1992; Cooper and McAuley-Hecht 1993; Camilleri et al. 1994) we predicted that complexation of these cyclic polysaccharides with surface protein residues might significantly affect the state of aggregation of protein in solution. The non-polar cavities of toroidal cyclodextrin molecules have a particular affinity for small non-polar groups and can both enhance the solubility and improve stability of such molecules in water. Cyclodextrins are finding increasing use as solubilizing agents and stabilizing excipients for protein drugs, including insulins (Brewster et al. 1991). We show here by calorimetric measurement of heats of dilution that the dissociation of bovine insulin in solution is significantly enhanced in the presence of various cyclodextrins. The energetics of this process are consistent with association of cyclodextrin molecules with insulin surface residues. Such a complexation might also bring about conformational changes in insulin which could contribute indirectly to the disaggregation process.

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Materials and methods

Bovine Zn-insulin was obtained from Sigma (I-5500) and used without further purification (trial fplc, gel filtration and dynamic light scattering experiments indicated no significant protein contaminants in these preparations). Protein solutions were prepared fresh daily in the appropriate buffer, and concentrations verified by UV absorbance measurements on diluted aliquots assuming a molar extinction coefficient (ε_{280}) of 5734 M^{-1} (Porter 1953; Gill and von Hippel 1989). Buffers used were 0.1 M glycine/HCl pH 2.5 or 0.1 M Na-phosphate pH 7.4, containing appropriate concentrations (by weight) of cyclodextrins where required. Hydroxypropyl-β-cyclodextrin (0.8 avg.subst.) and methyl-β-cyclodextrin (1.8 avg.subst.) were obtained from Aldrich, α-cyclodextrin was from Sigma Chemical Co.

Calorimetric dilution experiments were done using a Microcal OMEGA titration microcalorimeter following standard instrumental procedures at 25 °C with a 250 μl in-

jection syringe and 400 rpm stirring (Wiseman et al. 1989; Cooper and Johnson 1994). In a typical dilution experiment small aliquots (10–20 µl) of concentrated insulin, dissolved in buffer or buffer/cyclodextrin mix, were injected into the calorimeter reaction vessel (1.4 ml volume) containing the identical buffer mixture. Integrated heat pulse data, after correction for mixing controls done separately under identical conditions, were analysed by non-linear regression in terms of a simple monomer-dimer equilibrium model to give the apparent equilibrium constant (K_{diss}) and enthalpy of dissociation (ΔH_{diss} per mole dimer):

$Ins_2 \rightleftharpoons 2Ins$; $K_{diss} = [Ins_{tot}]^2/[Ins_2]$; ΔH_{diss}

where $[Ins_{tot}]$ represents the total free insulin monomer concentration. Other thermodynamic parameters were calculated from standard expressions:

$\Delta G^0 = -RT.\ln K$; $\Delta G = \Delta H - T.\Delta S$

Results and discussion

Dilution of a series of small aliquots of insulin into a larger volume of buffer in the microcalorimeter gives a sequence of endothermic heat pulses characteristic of molecular dissociation (Fig. 1 A). With successive injections, as protein concentration in the calorimeter cell increases, the magnitude of the heat uptake diminishes, presumably because the extent of dissociation decreases at the higher insulin concentrations. This gives rise to a typical heat of dilution curve (Fig. 1 B) which can be fit in terms of a monomer-dimer equilibrium model yielding K_{diss} and ΔH_{diss}. Thermodynamic data obtained in this manner under a range of conditions are given in Table 1. Qualitatively similar effects are seen both at pH 2.5, where the insulin concentrations in the injection syringe were typically 10 mg/ml, and at pH 7.4 where, because of the much lower solubility, insulin concentrations were only about 1 mg/ml, and dilution heats were concomitantly smaller. Consequently, only limited quantitative data are reported here for pH 7.4, though the overall trends are similar at both pH's.

Addition of cyclodextrins to the buffer mixture gives rise to two significant effects (Fig. 2): (i) insulin dilution heats become more endothermic, and (ii) the dilution curves become more attenuated, indicating greater dimer dissociation in the presence of cyclodextrins. This is confirmed by the apparent thermodynamic parameters in Table 1, and is consistent with cyclodextrin binding to the dissociated form of the insulin molecule, thereby shifting the equilibrium in favour of dissociated protein. Previous studies have shown that, at low pH, insulin is predominantly dimeric in solution at high concentrations (Blundell et al. 1972) and, in agreement with this, our preliminary dynamic light scattering measurements (not shown) at pH 2.5, under the same conditions as used for calorimetric measurements, give an estimated molecular weight in the region of 11–12 kDa. The heat of dilution data obtained

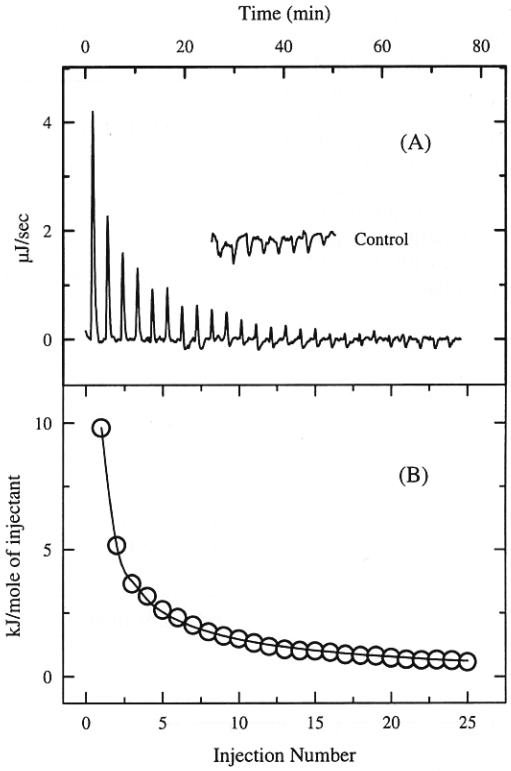

Fig. 1 A, B Calorimetric data for the endothermic dissociation of insulin dimers at pH 2.5. **A** Raw data for injection of insulin, 1.53 mM (8.8 mg/ml), 25×10 µl injections, into buffer at 25 °C, with control data showing the calorimetric response for blank buffer injections on this very sensitive scale; **B** Integrated injection heats, corrected for control heats and fit (solid line) to a dimer dissociation model with $K_{diss} = 12$ µM and $\Delta H_{diss} = 41$ kJ mol^{-1}

here are also consisted with a dimer dissociation model, and we use this for quantitative comparison. In the absence of cyclodextrins at pH 2.5 the insulin dimer dissociation constant (K_{diss}), obtained from non-linear regression analysis of dilution data, is around 12 µM and in agreement with previous determinations by other techniques (Blundell et al. 1972). Computer simulations (unpublished) of more complex oligomer models show sigmoidal dilution curves unlike anything observed so far here. At pH 7.4 the oligomeric state of insulin is less clear, and hexamers or higher oligomers almost certainly exist under the conditions used here. Nevertheless, the dilution data fit reasonably to a dimer model, and we use this for empirical comparison purposes.

The variation in apparent dissociation constant with cyclodextrin concentrations can be simulated, at least in part, by a simple (but probably unrealistic) model involving sequential binding of cyclodextrins to sites on the dissociated insulin. Assuming:

$Ins_2 \rightleftharpoons 2Ins$; $K_{diss,0} = [Ins]^2/[Ins_2]$; $\Delta H_{diss,0}$

and $Ins + C \rightleftharpoons Ins.C$; $K_1 = [Ins.C]/[Ins][C]$; ΔH_1

$Ins + Ins.C \rightleftharpoons Ins.C_2$; $K_2 = [Ins.C_2]/[Ins][Ins.C]$; ΔH_2

. . . and so on for sequential binding steps.

Table 1 Thermodynamic data for the dissociation of insulin dimers at 25 °C, determined from calorimetric dilution data in the presence or absence of cyclodextrins. Each experiment was normally repeated three times to give the mean values of apparent dissociation constants and enthalpies (K_{diss}, ΔH_{diss}) below, with standard deviations in parentheses

pH	Cyclodextrin	[CD] mM	K_{diss} μM	ΔH_{diss} kJ mol^{-1}	ΔG^{O}_{diss} kJ mol^{-1}	ΔS^{O}_{diss} J K^{-1} mol^{-1}
2.5	None	–	12 (1)	41.0 (3.5)	28.1	43
2.5	Methyl-β-	30	51 (13)	59.8 (3.9)	24.5	118
		50	88 (17)	64.7 (1.2)	23.1	139
		74	184 (19)	67.8 (5.1)	21.3	156
		99	284 (56)	72.5 (0.9)	20.2	175
		141	690 (99)	75.0 (0.9)	18.0	191
		199	1690 (21)	79.1 (2.1)	15.8	212
2.5	Hydroxypropyl-β	29	25 (12)	47.3 (6.2)	26.3	71
		50	24 (6)	54.7 (4.7)	26.4	95
		79	64 (24)	47.7 (4.7)	23.9	80
		120	70 (3)	53.1 (1.9)	23.7	99
		139	78 (4)	52.7 (1.9)	23.4	98
		209	152 (45)	58.3 (4.3)	21.8	123
2.5	α-	25	17 (3)	38.3 (0.8)	27.2	37
		48	41 (2)	34.0 (0.8)	25.0	30
		75	68 (8)	33.1 (1.2)	23.8	31
		97	67 (8)	29.4 (2.1)	23.8	19
7.4	None	–	nd[a]	nd	nd	nd
7.4	Methyl-β-	50	20	60	26.8	110
		150	190	88	21.2	224

[a] nd, calorimetric effects too weak to determine accurately

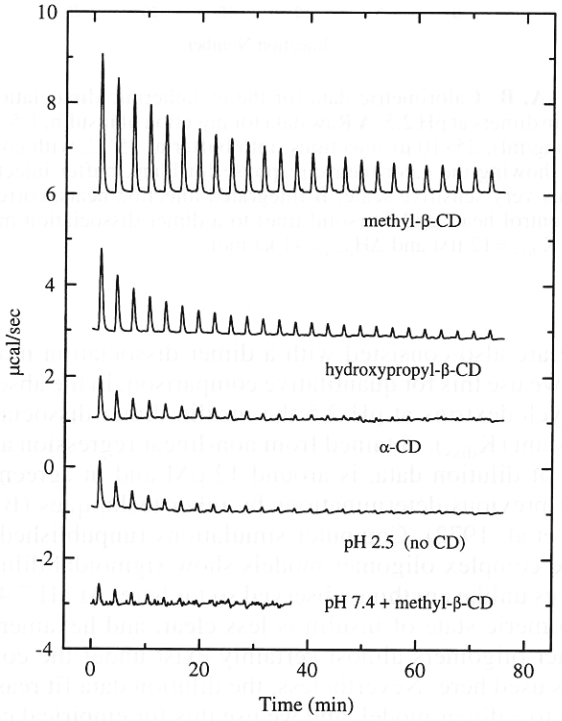

Fig. 2 Examples of raw calorimetric dilution data showing the effects of different cyclodextrins on insulin dissociation, all at pH 2.5 except where indicated. For comparison, cyclodextrin concentrations (when present) are all approximately 100 mM in this case

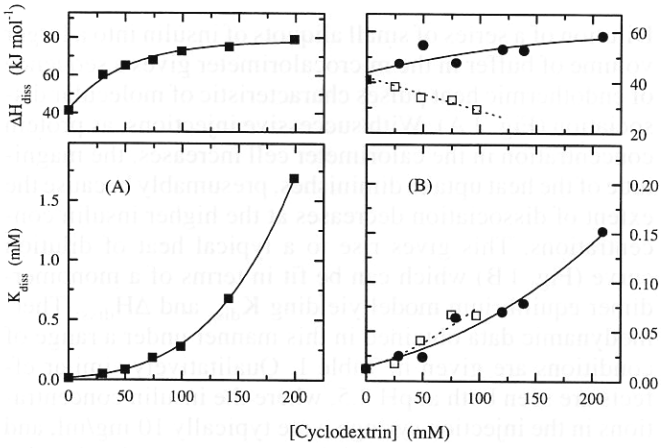

Fig. 3A, B Change in apparent dissociation constants (K_{diss}, lower panels) and enthalpies of dissociation of insulin (ΔH_{diss}, upper panels) at pH 2.5 with increasing cyclodextrin concentrations. In the lower panels the curves show the theoretical fits to a simple sequential binding model, using the parameters given in the text. **A** Methyl-β-cyclodextrin (*filled squares*). **B** Hydroxypropyl-β-cyclodextrin (*filled circles*) and α-cyclodextrin (*open squares*)

where C represents the cyclodextrin, the apparent dissociation constant may be written:

$$K_{diss} = [Ins_{tot}]^2/[Ins_2] = K_{diss,0}(1+K_1[C]+K_1K_2[C]^2+\ldots)^2$$

Despite uncertainties regarding the number of potential binding sites and their binding affinities, this polynomial expression does form a reasonable basis for empirical modelling of the observed effects of cyclodextrins on insulin dissociation thermodynamics. Non-linear regression analysis of experimental data for the variation of apparent dimer dissociation constant with cyclodextrin concentration (Fig. 3) indicates that two sequential binding sites are adequate for describing the data satisfactorily over the accessible concentration range. The numerical values obtained for the site-binding constants (K_1, K_2) are consistent with the relatively weak affinities expected on the basis of previous observations of interaction between cyclodextrins in solution and aromatic amino acid side chains

and similar groups (Cooper 1992; Cooper and MacNicol 1978; Horsky and Pitha 1994). For methyl-β-cyclodextrin, which shows the biggest effect, K_1 and K_2 are estimated to be about 20 and 6.5 M^{-1}, respectively, For the other cyclodextrins $K_1 \approx 10-15$ M^{-1}, with $K_2 < 5$ M^{-1}.

The enthalpies also show interesting variations. In the absence of cyclodextrins the dissociation of insulin oligomers is endothermic. Addition of α-cyclodextrin, in addition to encouraging oligomer dissociation, also makes this dissociation less endothermic in a manner consistent with the exothermic binding of α-cyclodextrins to exposed groups on insulin monomers after dissociation. In contrast, although methyl- and hydroxypropyl-β-cyclodextrins similarly induce oligomer dissociation, this dissociation is observed to be more endothermic. This suggests that the binding of these modified β-cyclodextrins to exposed insulin residues, although thermodynamically favourable, is endothermic and consequently entropy-driven. The nett effect is to increase the apparent insulin dissociation constant, whilst making the overall dissociation process more endothermic. The concomitant increase in dissociation entropy (ΔS^{O}_{diss}, Table 1) may arise from displacement of solvent/solvation layers from the insulin surface when the bulky modified β-cyclodextrins bind. It may also suggest an additional hydrophobic contribution to the cyclodextrin interaction arising from the methyl- and hydroxypropyl-substituent groups on the cyclodextrin ring. Such interactions may not be seen in model experiments involving just simple amino acids or analogues that lack the bulk/surface effects to be expected with sidechains on the insulin molecule.

It seems likely that cyclodextrins might have similar effects on the dissociation of other oligomeric systems, and further investigations are under way.

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